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ORIGINAL ARTICLE

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Triacylglycerols determine the unusual storage physiology of *Cuphea* seed

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Abstract Many species within the genus *Cuphea* (Lythraceae) produce seed with high levels of medium-chain fatty acids. Seeds of some *Cuphea* species lose viability when placed into storage at −18 °C. These species tolerate significant drying to 0.05 g/g and may, therefore, be intermediate in their storage characteristics. The thermal properties of seed lipids were observed using differential scanning calorimetry. Species with peak lipid melting temperatures ≥27 °C were found to be sensitive to −18 °C exposure while those with melting temperatures < 27 °C were able to tolerate low-temperature exposure. This relationship was determined by the triacylglycerol composition of the individual species. Sensitive species have high concentrations of lauric acid (C_{12}) and/or myristic acid (C_{14}). Species with high concentrations of capric (C_8) or caprylic acid (C_{10}) or with high concentrations of unsaturated fatty acids tolerate low temperature exposure. Potential damage caused by low temperature exposure can be avoided by exposing seeds to a brief heat pulse of 45 °C to melt solidified lipids prior to imbibition. The relationship between the behavior of triacylglycerols in vivo, seed storage behavior and sensitivity to imbibitional damage is previously unreported and may apply to other species with physiologies that make them difficult to store.

Keywords *Cuphea* · Desiccation tolerance · Freezing injury · Imbibition · Seed storage behavior · Triacylglycerol

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Abbreviations DSC: differential scanning calorimetry · RH: relative humidity

Introduction

Seeds from most oil-producing plant species contain long-chain fatty acids ($> C_{16}$). Many species within the genus *Cuphea* (Lythraceae), however, produce seed with high levels of medium-chain fatty acids (C_8–C_{14}) (Graham et al. 1981). These lipids are desirable in the food and chemical industries for a wide range of uses (Knapp 1993) and are currently obtained primarily from imported petrochemicals and tropical coconut and palm kernel oils. Some *Cuphea* species produce a relatively pure lipid, containing ≥85% of a single fatty acid, while others produce lipid mixtures with a range of fatty acids (Graham et al. 1981; Graham 1989; Knapp 1993).

Studies of seed physiology and dormancy have been conducted as part of the work toward developing and improving *Cuphea* germplasm for commercial use (Graham 1989; Knapp 1990; Widrlechner and Kovach 2000). Seed harvested from wild and semi-domesticated populations may be of poor quality as a result of *Cuphea's* indeterminate flowering habits and seed shattering. Dormancy and poor seed quality have confounded studies of stand establishment and storage behavior. For some species, stratification at 5 °C reduces dormancy and hastens germination (Graham 1989; Widrlechner and Kovach 2000). Viability of seeds from *C. viscosissima* was maintained for 4 years at ambient temperature (ca. 23 °C) and low moisture content (ca. 0.05 g H_2O/g dry mass; Widrelechner and Kovach 2000) and *Cuphea* species are routinely stored at 5 °C at the USDA–ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA (USA). However, viability of some *Cuphea* species is compromised when seeds are placed under the conventional storage temperature of −18 °C (C. Gardener and D. Kovach, NCRPIS, Ames, IA, USA, personal communication) recommended for orthodox seeds (IPGRI 1994).

A seed's ability to survive drying to low water contents, but inability to survive the combined stress of drying and sub-zero temperatures, has been reported for several genera, including *Azadirachta* (neem: Sacandé et al. 1998, 2000), *Carica* (papaya: Ellis et al. 1991a), *Citrus* (citrus: Hong and Ellis 1995), *Coffea* (coffee: Ellis et al. 1990a, 1990b; Hong and Ellis 1995; Eira et al. 1999), and *Elaeis* (oil palm: Ellis et al. 1991b) as well as for species native to the Brazilian Cerrado (Wetzel 1997), Western Australia (Merritt et al. 2003) and Hawaii (C. Walters, data not shown). A new category of storage behavior, called "intermediate," was recognized to account for these non-orthodox non-recalcitrant seeds (Ellis et al. 1990a, 1990b), but the basis of the physiology remains unknown. The IPGRI Electronic Seed Storage Behavior (ESSB) Compendium (Hong et al. 1996) cites 40 genera containing 60 species with intermediate storage behavior. A survey of the literature and our observations suggest that many of these species produce seeds with high oil content or unusual oil properties.

In this report, we document aspects of the storage behavior of *Cuphea* species and associate the damage incurred when some species are exposed to −18 °C to their lipid compositions. Thermal transitions occurring in the lipid components of seed were detected with differential scanning calorimetry (DSC) and allowed us to demonstrate that properties of the triacylglycerol storage lipids directly contribute to the observed differences among species in susceptibility to damage when exposed to −18 °C.

Materials and methods

Seeds of 35 *Cuphea* species were obtained from stored accessions at the USDA–ARS, National Center for Genetic Resources Preservation (NCGRP, formerly the National Seed Storage Laboratory). These accessions were stored for 6 months to 10 years in sealed foil-laminate bags at −18 °C at an adjusted moisture content of about 0.05 g H_2O/g dry mass. Germination history was obtained from records kept at NCGRP. Initial germination percentages varied among accessions. Seed supplies of all species in storage were limited; to allow further studies, an accession of *C. carthagenensis* (Ames 17845) was regenerated in greenhouses at the USDA–ARS North Central Regional Plant Introduction Station (NCRPIS) in Ames, IA (USA) during the summer of 2001 and shipped to NCGRP in Fort Collins, CO (USA) in October of 2001. Seeds of *C. carthagenensis* do not exhibit dormancy (Graham 1989) and the regenerated accession gave about 85% initial germination.

Viability assessments for *Cuphea* species consisted of germination assays and tetrazolium testing. To limit imbibitional damage, all samples were pre-hydrated over water for at least 18 h. Seeds were then placed on damp blotter paper in petri dishes at 25 °C with a 16/8 h light/dark cycle for up to 6 weeks. Seeds were scored as germinated when both the radicle and hypocotyl emerged. Tetrazolium tests (Peters 2000) were conducted to confirm viability after low-temperature treatments on four species (*C. aequipetala*, *C. carthagenensis*, *C. lanceolata* and *C. wrightii*). In these cases, seeds were imbibed overnight on moist blotter paper then cut and treated with a 1% solution of buffered 2,3,5-triphenyl-tetrazolium chloride. The embryos were observed approximately 8 h later for color development and uniformity of staining. Responses to low and high temperatures were measured by conducting germination assays on seeds that had been placed dry (ca. 0.05 g/g) at either −18 °C for at least 18 h or at 45 °C for at least 1 h.

Water contents were manipulated in *C. carthagenensis* seeds for desiccation tolerance and imbibition studies. To dry or slowly hydrate seeds, moisture content was adjusted by placing seed in different relative humidity (RH) chambers. RH was manipulated using a series of 10 different saturated salt solutions and ranged from 1 to 90%. Seeds were held in the chambers at ca. 23 °C for up to 2 weeks. More rapid imbibition was achieved by placing seeds on dampened blotter paper at 15 or 25 °C, depending on the experiment, for up to 90 min. Seed water content was measured gravimetrically with dry weights determined after drying for 96 h at 90 °C and is expressed on a dry-weight basis. Moisture contents for seeds held in the RH chambers were averaged from three replicates measured on different days and ranged from <0.01 to 0.2 g H_2O/g dry mass.

Lipid content and composition of various *Cuphea* species were obtained from the literature (Graham et al. 1981; Graham 1989) or measured directly if the information was unavailable or was inconsistent with DSC results (Table 2). To prepare samples for lipid extraction, about 0.05 g of seed was ground to a fine powder in a mortar and pestle. Lipids were extracted into chloroform–methanol in a 5-ml test tube using a modified Bligh and Dyer (1959) protocol. The total lipid fraction was re-dissolved in chloroform and fatty acid methyl-ester derivatives were prepared using 12% boron-trifluoride (Metcalfe and Schmitz 1961). The derivatives were then separated on a Perkin-Elmer 8500 chromatograph with an FID detector (Supelco Nukol 30 m, 0.25 mm i.d. fused silica capillary column; carrier gas: He; gas flow: 20 psi; injector and detector temperatures: 220 °C; oven temperature: 100 °C ramping to 190 °C at 10 °C min^{-1}). To determine if the triacylglycerols and membrane lipids had similar fatty acid compositions, neutral and polar lipid fractions of freshly regenerated *C. carthagenensis* were separated by loading the total lipid fraction, re-dissolved with a small amount of chloroform, onto a solid-phase extraction cartridge (Sep-pak Silica; Waters, Milford, MA, USA) previously flushed and primed with chloroform. The cartridge was slowly washed twice with chloroform to elute the neutral lipid fraction and then washed twice with methanol to elute the polar lipids. Fatty acids from each fraction were derivatized and analyzed by GC.

Thermal behavior of lipids in dry (0.05 g/g) whole seeds of 35 *Cuphea* species was measured with a Perkin-Elmer (Norwalk, CT, USA) DSC-7 calibrated for temperature with methylene chloride (−95 °C) and indium (156.6 °C), and for energy with indium (28.54 J g^{-1}; Vertucci 1992). Samples containing about 4 mg of seed were cooled from 20 °C to −100 °C at 10 °C min^{-1}, held for 1 min, and then warmed to 50 °C at the same rate. Onset temperatures of lipid transitions were calculated by Perkin-Elmer software from the intersection of the baseline and the tangent to the steepest part of the transition peak. Peak temperatures were recorded at the apex of the transition. Where several peaks were observed in a thermogram, the onset and peak temperatures are reported for the highest temperature transition. Theoretical melting temperatures for different crystalline structures (α, β, and β') can be calculated from the weighted average, based on fatty acid composition, of transition temperatures established for simple triacylglycerols (Small 1988). In this case, the theoretical melting temperature was calculated for the β' crystal forms of the lipids from the 35 species observed using DSC.

Results

Drying *Cuphea* seeds to a moisture content of 0.05 g/g (RH≃25% at 22 °C) did not affect germination for species studied here (data not shown). The limits of tolerance to drying were further tested in *C. carthagenensis* seeds dried to water contents as low as 0.005 g/g (RH≃1%; Fig. 1). Average germination for all moisture treatments was 77% with a standard deviation (SD) of 9.5% and there was no significant change with water

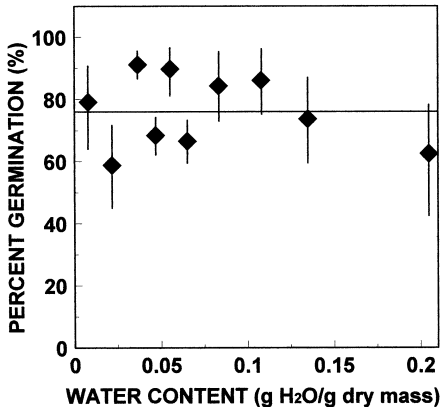

Fig. 1 Germination of freshly harvested *Cuphea carthagenensis* seed dried to a range of moisture contents from 0.01 to 0.20 g H_2O/g dry mass. Germination remained high and constant across the full range of moisture contents and averaged 77%. Data points and error bars represent the mean percent germination and SD of three replicates, respectively

content ($r^2 = 0.04$ and $P = 0.60$). These results confirm that *C. carthagenensis* seeds can tolerate extreme desiccation, at least for the 2-week period observed in this study, and therefore fit the criterion of most orthodox seed (Roberts 1973; Walters et al. 2002).

Tolerance to low temperature was evaluated during routine germination testing of *Cuphea* accessions from 15 species that had been held for 10 years at NCGRP under conventional storage conditions of −18 °C and a moisture content of about 0.05 g/g (Table 1). A large reduction in germination was noted in seven species, with some accessions giving 0% germination after storage. No change or only minor changes in germination was observed in five species. Germination increased after the storage treatment in accessions of three species and is attributed to a reduction in dormancy (Widrlechner and Kovach 2000). Based on these results, we divided *Cuphea* species into two categories: (i) those that are sensitive to low-temperature storage with germination reduced by at least 80% from the initial germination percentage and (ii) those that are tolerant of low-temperature storage with germination reduced no more than 20% from the initial germination percentage. This dichotomy was applied to 16 additional *Cuphea* species that were stored at −18 °C for about 6 months. A combined list of species and measured tolerance to −18 °C storage is given in Table 2. Of the 31 species for which germination records were available, 13 were considered to be sensitive to −18 °C storage. Germination of recently harvested *C. carthagenensis* seed decreased from 83 ± 6% for samples held at 5 °C to 0% germination for samples given an 18 h exposure to −18 °C, demonstrating that, for this species at least, damage was incurred upon a brief exposure to −18 °C.

Table 1 Effect of low temperature (−18°C) exposure on germination of 15 *Cuphea* species. Species divide into two groups on the basis of germination following low-temperature treatment. Mean germination ± SD are shown

Species	Number of accessions	Germination (%)	
		Before −18 °C	After −18 °C
C. aequipetala	4	87 ± 2	9 ± 11
C. aperta	1	28	0
C. calophylla	2	71 ± 2	1 ± 1
C. cathagenensis	7	33 ± 31	1 ± 1
C. parsonsia	1	74	0
C. tolucana	7	70 ± 13	1 ± 1
C. wrightii	10	55 ± 23	8 ± 7
C. angustifolia	2	55 ± 9	37 ± 13
C. hookeriana	2	95 ± 3	93 ± 3
C. koehneana	2	42 ± 15	48 ± 14
C. lanceolata	13	85 ± 6	78 ± 8
C. lutea	2	63 ± 9	81 ± 7
C. paucipetala	1	35	72
C. procumbens	3	46 ± 25	37 ± 30
C. viscosissima	31	20 ± 8	78 ± 13

Seeds of *Cuphea* species had a diverse array of lipid compositions. From the literature, lipid content can range from 16 to 42% of the total seed weight (Graham 1989), primarily from storage lipids (Table 2). Several species produced fairly pure fractions with at least 80% of a single fatty acid. For 28 of the 36 species observed, 75% of the fatty acids fell within a two-carbon chain length, i.e. C_8 and C_{10}, C_{10} and C_{12}, or C_{12} and C_{14}. Four species, *C. racemosa*, *C. mimuloides*, *C. decandra* and *C. gaumeri* had a high proportion of fatty acids with 16 or more carbons and from 34–76% unsaturated fatty acids. The triacylglycerol fraction of *C. carthagenensis* seeds contained predominantly lauric (C_{12}) and myristic (C_{14}) fatty acids (Table 3, columns 1–4). However, the polar lipid fraction was composed predominantly of longer chain, unsaturated fatty acids, typical of the lipid compositions of more commonly used oilseeds.

A comparison of lipid composition among *Cuphea* species with sensitivity to −18 °C storage suggests that seeds are susceptible to damage on exposure to −18 °C if they contain lauric acid (C_{12}) and/or myristic acid (C_{14}) in proportions ≥58% of total fatty acids (Table 2). Seeds containing either high proportions of caprylic acid (C_8) and capric acid (C_{10}) or longer-chain unsaturated fatty acids ($\geq C_{16}$) show no apparent damage from −18 °C exposure. A ratio of $(C_{12}+C_{14}):(C_8+C_{10}) > 2$ corresponds to intolerance of −18 °C and can be used to predict susceptibility for species for which germination was not tested. *Cuphea racemosa*, *C. ignea*, *C. inflata*, and *C. crassifolia* are predicted to store safely at −18 °C while *C. micrantha* is expected to be damaged from −18 °C exposure.

The strong correspondence between lipid composition and tolerance of *Cuphea* species to −18 °C prompted a study of the thermal behavior of *Cuphea* lipids to determine if phase transitions occurred in the temperature range for which damage was seen. Lipid transitions were observed from DSC analyses of seeds from 35 *Cuphea* species. Representative scans are given in Fig. 2. Lipid crystallization events for all species occurred

Table 2 Total fatty acid composition of selected *Cuphea* species in relation to seed tolerance of −18 °C exposure. Fatty acids with 16 or fewer carbons are saturated and percentages of unsaturated fatty acids from 18-carbon or longer fatty acids are combined. Species high in C_{12} and C_{14} in comparison to C_8 and C_{10} do not tolerate −18 °C exposure. (*a*) or (*b*) following a species name refers to a species for which the fatty acid composition in the literature (*a*) was inconsistent with our laboratory analyses and for which we analyzed additional samples (*b*). Germination results are designated by *nd* when seed was unavailable for testing or the appropriate accession was unknown (*a*). References: *1* Graham et al. 1981; *2* Graham 1989; *3* USDA–ARS, National Center for Genetic Resources Preservation (NCGRP). Labels in final column refer to species labels on Fig. 3

Species	C8	C10	C12	C14	C16	C18	≥C18 unsat	(C12 + C14)/(C8 + C10)	Tolerant of −18 °C	Ref.	Label
C. racemosa	0	0	0.1	0.2	15.3	1.7	82.7	≈0	nd	1	–
C. mimuloides	0	0	25.0	10.0	20.0	3.0	40.0	≈0	Yes	1	–
C. painteri	65.0	24.0	0.2	0.4	2.8	0.4	7.2	0.01	Yes	1	PA
C. leptopoda	1.1	87.3	1.5	0	2.3	0.3	6.8	0.02	Yes	2	–
C. ignea	0.9	87.1	1.2	0.6	3.1	0.2	7.0	0.02	nd	1	–
C. koeheana	0.1	91.6	1.5	0.6	1.3	0.3	4.4	0.02	Yes	2	–
C. inflata	0.7	86.4	1.6	0.4	2.1	0.5	7.8	0.02	nd	1	IN
C. crassiflora	1.4	87.2	1.7	0.4	2.3	0.6	6.2	0.02	nd	1	CR
C. llavea	1.5	87.5	1.6	0.7	1.9	0.4	6.0	0.03	Yes	1	–
C. paucipetela	1.2	87.4	2.0	0.8	1.9	0	5.8	0.03	Yes	1	–
C. lanceolata	0.6	83.2	2.1	2.0	3.4	0	8.0	0.05	Yes	1	LA
C. viscosissima	9.1	75.5	3.0	1.3	3.1	0.3	7.1	0.05	Yes	2	–
C. procumbens	0.4	80.1	2.7	1.5	4.5	0.3	8.8	0.05	Yes	1	–
C. hookeriana	50.2	25.4	3.6	1.0	7.1	0.7	8.9	0.06	Yes	1	HO
C. angustifolia	0	80.4	2.6	2.9	3.4	0.3	10.7	0.07	Yes	1	–
C. lophostoma	0.9	81.1	2.4	4.1	2.9	0.5	6.2	0.08	Yes	1	–
C. calcarata (a)	0	64.3	6.3	6.9	6.5	0.9	14.6	0.21	nd	1	–
C. decandra	0	1.3	0.3	0.3	29.6	2.4	65.3	0.69	Yes	1	DE
C. gaumeri	5.0	7.3	1.1	10.2	1.4	14.9	60.0	1.46	Yes	3	GA
C. heterophylla	2.6	32.8	47.7	6.5	2.0	0.5	8.6	1.50	Yes	1	HE
C. glutinosa (b)	4.4	30.9	49.6	4.7	1.9	0	8.0	1.53	Yes	3	GLU
C. lutea	0.4	29.4	37.7	11.1	4.1	0.7	6.9	1.64	Yes	1	LU
C. calcarata (b)	3.8	20.2	54.5	8.7	3.5	0.5	8.7	1.75	Yes	3	CALC
C. wrightii	0	29.4	53.9	5.1	2.3	0.4	7.7	2.01	No	1	WR
C. aequipetala	24.6	1.3	1.8	56.0	6.6	0	8.5	2.23	No	1	AE
C. tolucana	0	23.0	63.3	4.5	1.8	0	6.9	2.95	No	1	TO
C. palustris	19.7	1.4	2.0	63.7	6.7	0	5.9	3.11	No	1	PAL
C. aperta	0	16.2	76.2	3.0	1.2	0	1.9	3.62	No	1	AP
C. glutinosa (a)	0.5	17.6	64.8	3.9	2.3	0.4	10.3	3.80	nd	2	–
C. glossostoma	0	17.3	59.4	9.2	3.1	0.5	10.0	3.95	No	1	GLO
C. laminuligera	0	17.1	62.6	9.5	2.8	0.2	5.5	4.22	No	1	LA
C. calophylla	0.1	5.0	85.0	6.8	3.0	0.1	3.4	7.23	No	2	CALO
C. carthagenensis	0	8.0	62.5	13.4	3.5	0	3.9	8.12	No	2	–
C. parsonsia	0	7.9	73.9	4.4	0.9	0	6.0	9.91	No	1	PAR
C. hyssopifolia	0.2	7.3	78.5	4.8	1.5	0.2	3.8	11.11	No	1	HY
C. carthagenensis	0	4.5	65.5	18.7	4.1	1.0	3.9	17.91	No	3	CAR
C. micrantha	0	2.4	53.2	17.8	6.3	0.8	12.9	29.58	nd	1	MI
C. ericoides	0.3	1.5	41.9	21.1	6.7	1.4	12.2	35.00	No	3	ER

during cooling at onset temperatures ranging from +16 °C to −59 °C (Fig. 2a, c). The size of the transition, the number of crystallization peaks and the peak temperature varied among species, although crystallization onset temperature was roughly correlated to fatty acid composition. Seeds with high capric acid (C_8), caprylic acid (C_{10}), or either high lauric (C_{12}) or myristic (C_{14}) acid contents had average onset temperatures of −40, −24 and −3 °C, respectively.

As expected for lipids, transition behavior upon warming *Cuphea* seeds is complex (Small 1988; German and Simoneau 1998; Fig. 2b, d). Exotherms that occur upon warming, termed recrystallization events, were observed in most species at temperatures between −50 °C and +5 °C. The temperature of melting transitions varied among species and was consistent with differences in the fatty acid composition. Species highest in caprylic acid had melting onset temperatures below −5 °C, those high in capric acid averaged an onset temperature of 11 ± 6 °C (mean ± SD, $n = 13$) and seed high in either myristic or lauric acid had an onset temperature averaging 22 ± 9 °C (mean ± SD, $n = 16$). The melting temperatures for different crystal forms of simple triacylglycerols are presented in Table 3 (Malkin 1954; Small 1988) and can be used to roughly predict thermal behavior. For *C. carthagenensis* a weighted average, based on fatty acid proportions, of the temperatures for β' transitions gave a predicted melting temperature of 35 °C. DSC analysis showed actual peak melting temperature of 33 °C for this species (Fig. 2b). Similarly calculated melt temperatures for other *Cuphea* species based on a weighted average of the β' crystal form correlated to the melt temperatures measured using DSC ($r^2 = 0.73$, slope = 1.05, SE of coefficient = 0.05;

Table 3 Composition of neutral and polar lipid fractions extracted from *C. carthagenensis*. Melting temperatures for simple triacylglycerols, which have the same fatty acid on each chain, and diacylglyceride–phosphotidylcholines (in excess water) are given from Small (1988)

Fatty acid	Formula	Composition of lipid fractions (%)		Melting temperature (°C)			
				Triacyglycerol			Phospholipid
		Neutral	Polar	β	β'	α	
Caprylic	C8	0	0	8	−21	−25	–
Capric	C10	6	0	32	18	−15	–
Lauric	C12	61	6	47	35	15	≈ 0
Myristic	C14	22	6	57	47	33	≈ 23
Palmitic	C16	4	22	66	57	45	≈ 42
Stearic	C18	0	10	73	64	55	≈ 54
Oleic	C18:1	2	20	6	−12	−32	≈ −20
Linoleic	C18:2	4	33	−13	−23	−43	≈ −30
Linolenic	C18:3	0	3	−24	−34	−45	≈ −35
				Average melting temperature weighted by fatty acid composition			
≤ C10	–	6	0	15	35	45	≈ 1
C12 + C14	–	83	12				
≥C16	–	11	88				

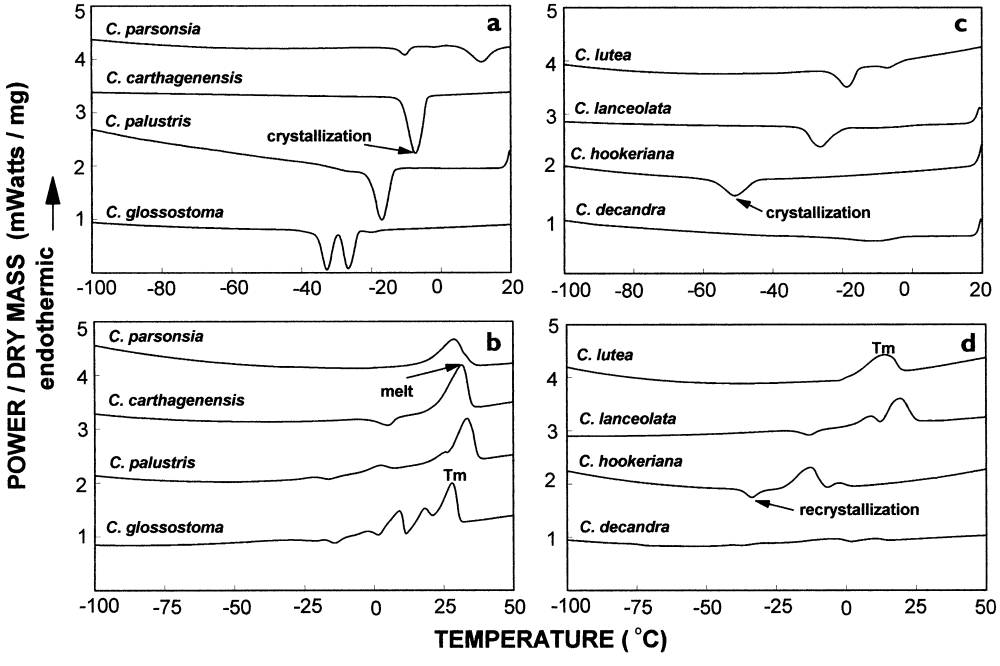

Fig. 2 Cooling (**a, c**) and warming (**b, d**) thermograms of representative *Cuphea* species measured using DSC. Species sensitive to storage at −18 °C (**a, b**) have a $(C_{12}+C_{14})$:(C_8+C_{10}) ratio >2, while species that tolerate −18 °C storage (**c, d**) have a ratio <2. Representative crystallization transitions, which occur on cooling, are indicated, as are representative recrystallization and melting transitions, which occur on warming. *Tm* Transition temperature at the highest melting peak

Fig. 3). The correlation coefficient increased if the temperatures of β crystal transitions (Table 3) were considered for species with high concentrations (≥85%) of a single fatty acid or if the temperatures of the α crystal transitions (Table 3) were considered for species containing unsaturated fatty acids (data not shown).

In Fig. 4, the onset temperature of the endothermic peak is plotted against the ratio of fatty acid chain lengths for the *Cuphea* species studied. Species with $(C_{12}+C_{14})$:(C_8+C10) ratios of less than 2 (Table 2) had peak temperatures ≤ 27 °C (mean and SD = 14.4 ± 9.7 °C), and those with higher ratios had peak temperatures >27 °C (mean and SD = 32 ± 3.6 °C). Those species with peak temperatures >27 °C (open symbols in Fig. 4) were damaged by exposure to −18 °C (Tables 1, 2). Warming to ambient room temperature (ca. 23 °C) was therefore sufficient to melt lipids in seeds that were tolerant of −18 °C, but was not sufficient to melt lipids in seeds that were intolerant to −18 °C exposure.

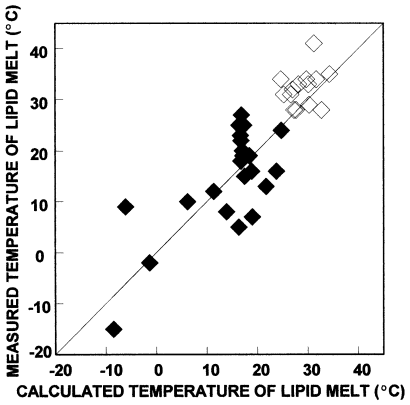

Fig. 3 Peak temperature of the lipid melt as determined by DSC plotted against the calculated melt temperature for the β' crystal form. The melting temperature for the β' crystal form was calculated from the equation: $\sum_{n=8}^{18}$ (% of fatty acid n)$*$ (melt. temp. of TAG with fatty acid n). ◆ Species not damaged by −18 °C, ◇ species damaged by −18 °C

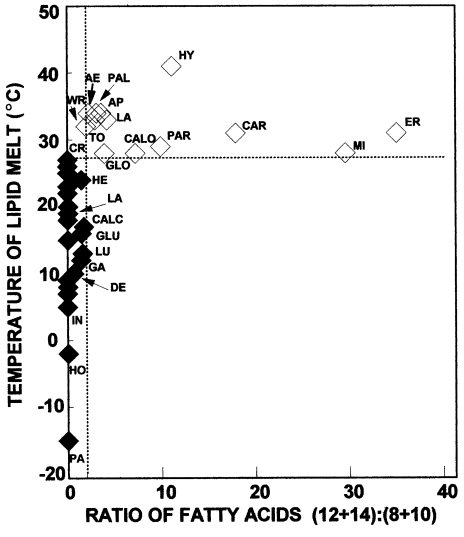

Fig. 4 Peak temperature of the final (highest temperature) lipid melt observed from DSC scans as a function of the ratio of fatty acid content $(C_{12}+C_{14})$:(C_8+C_{10}) for seed from 35 *Cuphea* species. ◆ Species not damaged by −18 °C, ◇ species damaged by −18 °C. Labels indicate selected species as listed in Table 2

Comparison of lipid phase behavior and seed survival following exposure to −18 °C suggests that seeds were damaged if their lipids were in a crystalline state (unmelted) when the seeds were imbibed at 25 °C. To test this hypothesis, seeds of six selected species that were sensitive and seven species that were not sensitive to −18 °C storage (Table 2) were removed from storage and immediately warmed to 45 °C to melt all lipids (Fig. 2b, d) before the seeds were imbibed and germinated at 25 °C (Fig. 5). This heat pulse increased germination of seed lots previously scored as dead by at least 40% (*C. ericoides*) and as much as 90%

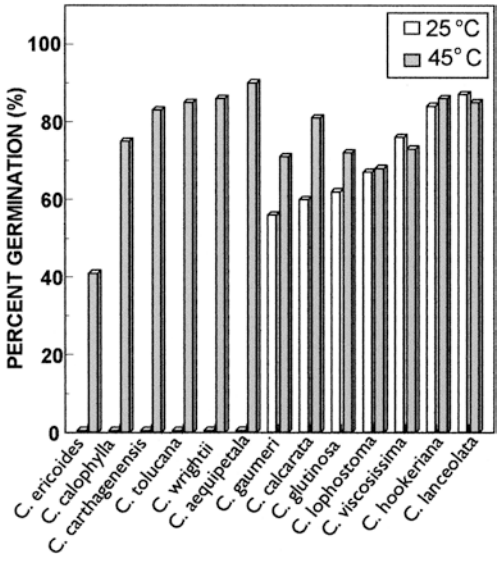

Fig. 5 Germination of representative *Cuphea* species stored at −18 °C and imbibed at 25 °C either with or without a 45 °C pulse before imbibition. Species with >50% germination following imbibition at 25 °C with no heat pulse are representative of species that tolerate −18 °C storage. Species with 0% germination following imbibition at 25 °C with no heat pulse are representative of species that are sensitive to −18 °C storage

Table 4 Percent germination of*C. lanceolata* seeds imbibed at 15 °C following exposure to −18 °C and various pre-imbibition treatments. Seeds were imbibed for 5 h and then transferred to 25 °C according to standard germination procedures

Temperature before imbibition	Imbibition temperature	% Germination
25	25	88
25	15	85
45	15	90
15	15	0

(*C. aequipetala*) with an average increase of 77%. For species showing insignificant sensitivity to −18 °C (germination ≥ 50% following storage at −18 °C), the brief exposure to 45 °C resulted in a 0–20% increase in germination and mean germination for this category of seeds increased from 70 to 76%.

A further test of the requirement for fully melted lipids prior to imbibition was made using *C. lanceolata*, a species that does not demonstrate sensitivity to −18 °C storage when warmed to room temperature prior to germination (Fig. 5). Seeds of *C. lanceolata* were removed from storage and either immediately imbibed at 15 °C or treated with a 45 °C pulse prior to imbibition at 15 °C. After 5 h imbibition, all seeds were moved to 25 °C for germination. The lipids of *C. lanceolata* were only partially melted at 15 °C since melting transitions began at 13 °C and peaked at 19 °C (Fig. 2d). Seeds that were not given a 45 °C pulse before imbibition at 15 °C did not germinate, while seeds receiving the brief high-temperature treatment germinated normally (Table 4).

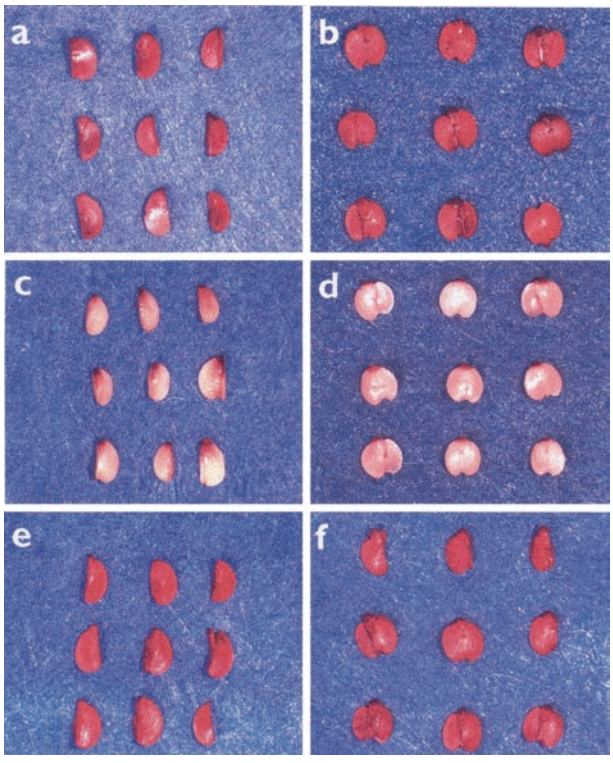

Fig. 6 Tetrazolium testing of a low temperature tolerant species (*C. lanceolata*, Fig. 5; **a, c, e**) and a sensitive species (*C. carthagenensis*, Fig. 5; **b, d, f**). Panels **a, b** *C. lanceolata* seeds removed from −18 °C storage and warmed to 25 °C prior to testing and freshly harvested *C. carthagenensis* seeds, respectively. Panels **c, d** seeds removed from −18 °C and warmed only to 15 °C prior to hydration at 15 °C. Panels **e, f** Seed removed from −18 °C and warmed to 45 °C for 1 h prior to hydration at 15 °C

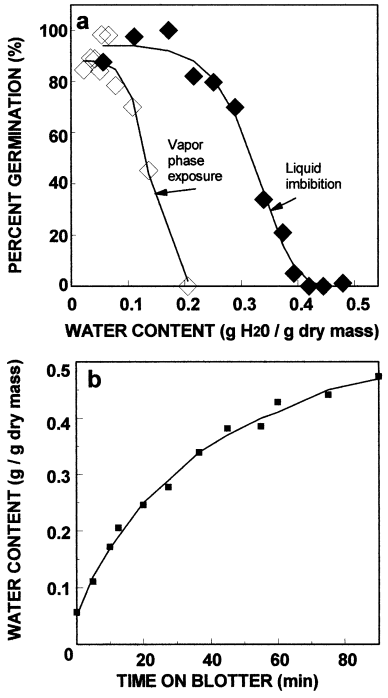

Fig. 7a, b Survival of *C. carthagenensis* seed exposed to −18 °C overnight, warmed to 25 °C, and exposed to a 45 °C heat pulse at various stages of imbibition. Seeds were either slowly hydrated over saturated salt solutions for 48 h (◇) before receiving the heat treatment or were rapidly hydrated by soaking on damp blotter paper (■) for up to 90 min prior to the heat treatment

Tetrazolium viability staining of seeds from *C. lanceolata* and *C. carthagenensis* confirmed the requirement that lipids be fully melted before imbibition (Fig. 6). Seeds of *C. lanceolata* warmed to 25 °C and newly harvested seeds of *C. carthagenensis* gave 82% and 90% viability, respectively, based on strong color development (Fig. 6a, b, respectively). Staining was weak in the cotyledons for both species when seeds were exposed to −18 °C and imbibed at 15 °C, corresponding to 0% germination for both species (Fig. 6c, d). Color development was evident in both species following a 1-h exposure to 45 °C before imbibition at 15 °C with viability assessed as 88 and 100% for *C. lanceolata* and *C. carthagenensis*, respectively (Fig. 6e, f). The embryonic axes from seeds imbibed at 15 °C without pre-exposure to 45 °C stained (Fig. 6c, d), but the cotyledons did not and the seeds did not germinate, suggesting that the condition of the storage reserves is important to the germination of the entire seed.

The above experiments demonstrate that *Cuphea* seeds in storage at −18 °C may be viable, but can be killed during germination or hydration prior to tetrazolium testing if the lipids are not fully melted when the seeds are imbibed. The interaction between hydration of seeds and the condition of lipids (melted versus crystalline) was explored by imbibing *C. carthagenensis* seeds at 25 °C on damp blotter paper or in RH chambers and providing a 1-h 45 °C pulse at different times during imbibition. The water content of seeds that were imbibed on damp blotter paper increased from 0.05 g/g to 0.5 g/g in 90 min (Fig. 7b). These seeds showed no apparent damage if they received the 45 °C pulse within the first 15 min of imbibition (water content < 0.2 g/g; Fig. 7a). Heat treatments after longer imbibition times or higher water contents gave reduced survival, with all seeds killed by imbibition of 1 h or more (water contents ≥ 0.4 g/g). Seed that was hydrated slowly in RH chambers showed no damage if water content levels were at or below 0.08 g/g when the seeds received a 45 °C heat pulse. Damage progressively increased for seeds at higher water contents and by 0.2 g/g (92% RH) all seeds were dead following the 45 °C heat pulse and subsequent imbibition.

Discussion

In this paper, we report an unusual seed physiology for species of *Cuphea*. To our knowledge this behavior, linked to the melting behavior of triacylglycerols, has not been reported previously, though it is relevant to general studies of seed storage behavior and imbibitional damage.

Cuphea species produce an unusual compositional array of medium-chain fatty acids in the storage lipids of seeds (Tables 2, 3). The crystallization and melting temperatures of lipids composed of short-chain saturated fatty acids are lower than those composed of longer-chain saturated fatty acids, but higher than those for longer-chain unsaturated fatty acids (Table 3; Small 1988). Crystalline structures of triacylglycerols are polymorphic and the α and β' phases are most likely formed in mixtures such as in seeds (Small 1988; German and Simoneau 1998;) although the β phase may be found in seed with a relatively pure fatty acid component. The close correspondence of lipid melting temperatures calculated for β' transitions (Table 3, Fig. 3) to those measured with DSC (Figs. 2, 4) strongly implicates β' crystals as the predominant crystal formed during the low-temperature treatments used in these experiments (C. Bailly, Universite Pierre et Marie Curie, Paris, France, personal communication). *Cuphea* seeds, stored at −18 °C, were killed if the temperature of the lipid melt was greater than the imbibition temperature (Figs. 3, 4, open symbols; Table 4). Damage could be prevented by heat treatments that melted the lipids before seeds were exposed to water (Figs. 5, 6, 7). Thus, sensitive *Cuphea* seeds were not damaged by exposure to −18 °C per se, but by hydration following the low-temperature exposure.

The apparent damage to *Cuphea* species upon exposure to −18 °C may lead to their classification as seeds with storage behavior intermediate to orthodox (desiccation and low temperature tolerant) and recalcitrant (desiccation and low temperature intolerant). However, as demonstrated by *C. carthagenensis*, some species exhibit extreme desiccation tolerance with the ability to survive drying to 1% RH (Fig. 1), which is unusual for species considered to be in the intermediate category (Ellis et al. 1990a, 1991a; Eira et al. 1999; Dussert et al. 1999; Sacandé et al. 2000). In addition, damage from exposure to −18 °C can be prevented or eliminated by a high-temperature treatment sufficient to melt the lipids (Figs. 4, 5, 6; Table 4). The simple warming treatment was sufficient to give similar germination percentages for cold-sensitive species (77%) and cold-tolerant species (76%). It is unknown whether a similar heat pulse can revive other seeds classified in the intermediate category, although an earlier study suggested that imbibition of neem (*Azadirachta indica*) at temperatures ≥35 °C was beneficial (Sacandé et al. 1998). Germination percentages of *Cuphea* seeds stored for 10 years at −18 °C and about 0.05 g/g were similar to freshly harvested seeds, if stored seeds received a 45 °C pulse prior to germination (compare germination percentage in Fig. 4 with initial values listed in Table 1). This suggests that *Cuphea* species can be conserved in seed banks for extended times.

Damage to some *Cuphea* species exposed to −18 °C was incurred during the initial stages of water uptake in seeds (Fig. 7a), but the circumstances of the damage do not conform to current views of imbibitional damage. In previous reports, cold water (e.g. Hobbs and Obendorf 1972), speed of hydration (Vertucci 1989), and seed aging (van Bilsen et al. 1994; McKersie et al.1989; Sacandé et al.1998; Pammenter and Berjak 1999) exacerbate imbibitional damage which may be ameliorated by pre-hydration at high humidity (Hobbs and Obendorf 1972; Crowe et al. 1989a, 1989b; Hoekstra et al. 1992, 1999) or by brief exposure to high temperatures before imbibition (Crowe et al. 1989a, 1989b; Hoekstra et al. 1992, 1999). Imbibitional damage is believed to result from changes in membrane lipids from the gel to the liquid crystalline phase as dry seeds become wet (Crowe et al. 1989a, 1989b; Hoekstra et al. 1992, 1999; Sacandé et al. 1998). In this study, however, damage to *C. carthagenensis* seeds is exacerbated by slow hydration over high humidity (Fig. 7a) and damage correlates with phase behavior of the triacylglycerols (Figs. 4, 5, 6; Table 4) rather than the polar membrane lipids which have lower melting temperatures due to a high linoleic acid content (Table 3). The transition temperature of membrane lipids is predicted to be about 1 °C, based on weighted average of phosphatidylcholine–diacylglycerol mixes given in Table 3.

We do not know why the condition of the triacylglycerols at the onset of imbibition is critical to seed survival. The behavior of triacylglycerols in vivo is poorly understood, but appears to be important to seed quality (Vertucci 1992; Leprince et al. 1998; C. Bailly, personal communication). We show that embryonic axes exposed to −18 °C survived without a pre-imbibition heat pulse, but damage to cotyledons resulted in seed death (Fig. 6c, e). A similar observation was made for high-stearate rapeseed (Thompson and Li 1997). Localization of damage in the cotyledons and involvement of triacylglycerol phase behavior suggest that damage resulted in a disruption in mobilization of food reserves. Mobilization of lipids in large oil bodies is less efficient than in smaller ones and oleosins are believed to stabilize the size of oil bodies to help facilitate breakdown of triacylglycerols (Huang 1992; Herman 1995; Murphy et al. 2001). Oil bodies in seeds with low levels of oleosins tend to coalesce during imbibition, resulting in disruption of the cellular structure of the cotyledons (Leprince et al. 1998). Leprince and colleagues' experiments used recalcitrant seeds of cacao, whose almost pure component of palmitic and stearic acids would likely be crystalline under the conditions of the experiment (Hartel 1998). If the results presented for *Cuphea* species are related to those reported for cacao, we would expect a low oleosin content in *Cuphea* seeds and coalescence of oil bodies in seeds imbibed without adequate heat treatment. Conversely, we would expect the coalescence of cacao or high-stearate rapeseed oil bodies to be prevented by heat treatments (to about 60 °C) before seeds are imbibed.

Cuphea germplasm can be conserved by storing seeds under standard conditions (IPGRI 1994). Seeds of some species must be warmed to 45 °C before imbibition to prevent lethal damage. The heat pulse melts

triacylglycerols in *Cuphea* species that contain high levels of lauric (C_{12}) and myristic (C_{14}) fatty acids. This paper reports a new link between triacylglycerol behavior in vivo, seed storage behavior and sensitivity to imbibitional damage. This connection may be applicable to many seed species that are currently not stored in genebanks because of their difficult physiologies.

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